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Adenovirus-Receptor Interaction With Human Lymphocytes

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Lymphocytes play a key role in cell-mediated immunity and are host cells for several viral and bacterial pathogens. Their importance in adenovirus (Ad) infections is not yet fully understood. The initial event, the attachment of Ad to lymphocytes and their subsets, was examined using flow cytometry. The study included analysis of stimulated T cells in binding assays with FITC-labeled Ad fiber. The results confirm that native peripheral lymphocytes express very small amounts of Ad receptors. Stimulation with PHA and interleukin 2 induced the expression. The presence of Ad DNA as a sign of internalization in stimulated cells was demonstrated using the polymerase chain reaction. The findings suggest that lymphocytes after stimulation can turn into target cells for Ad. This is particularly important if there are indications for persistence of Ad, and in the case of immunocompromised patients severe, life-threatening diseases can develop. ***J. Med. Virol. 51:252–257, 1997.*** © 1997 Wiley-Liss, Inc.

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INTRODUCTION

Adenoviruses (Ad) replicate in a variety of targets and are associated with several clinical syndromes, such as respiratory, gastrointestinal, and ocular diseases. Although Ad of subgroup C usually cause self-limited diseases in the immunocompetent host, they can develop severe, life-threatening infections in the immunocompromised [Hierholzer, 1992]. The ability of Ad to establish persistent infections has been recognized [Neumann et al., 1987], but the mechanism of generating such persistent infections in humans is poorly understood. In viral infections, lymphocytes play an important role as key cells of the immune system, but there is little information on Ad interaction with human lymphocytes. Incidence for limited binding capacity of the Ad to these cells and their infection was reported [Horvath and Weber, 1988; Silver and Anderson, 1988; Huang et al., 1995]. Information on virus interaction with the receptor on lymphocytes is not only helpful for the elucidation of the pathogenesis but it also permits us to find new therapeutic concepts [Mentel et al., 1993, 1996].

In the present study, the interaction between Ad and lymphocytes was investigated using fiber protein. The fiber as a subunit of the Ad is a trimeric protein which enables Ad attachment to cells [Stouten et al., 1992; Fender et al., 1995]. The aim of the study was to find out under which conditions lymphocytes are able to bind Ad fiber protein and which subpopulations are involved and whether the first step of adsorption is followed by penetration into lymphocytes, leading to virus persistence.

MATERIALS AND METHODS

Cells and Virus

Continuous passages of Fogh and Lund cells (FL cells), ATCC, CCL 62, were undertaken in Eagle's minimal essential medium (MEM) containing 7% heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin G, and 100 μg/ml of streptomycin. Cells were subcultured every 5–6 days. Ad type 2 was propagated in FL cells using Eagle's MEM containing 2% FCS. Virus-infected cells were frozen and thawed three times, and after titration aliquots of viral stocks were stored at −80°C.

Ad Fiber Preparation and Labeling With Fluoresceine Isothiocyanate (FITC)

Fiber protein of Ad type 2 was isolated by extraction of fibers from infected FL cells, followed by purification using affinity chromatography. Briefly, extraction was done by a freeze-thaw cycle three times. After centrifugation (10 min, 10,000 rpm), the supernatant was

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loaded on the column filled with sepharose ligated to antibodies against Ad 2 fiber. Antibodies were prepared by immunizing rabbits with fibers of Ad 2 purified by diethylaminoethanol (DEAE) chromatography. For elution of fiber protein 4 M guanidinium hydrochloride in phosphate-buffered saline (PBS) was used. After dialysing against PBS, the fiber was concentrated to 2–3 mg/ml by ultrafiltration using Centriprep 30 (Amicon, Beverly, MA). Analysis of preparation on polyacrylamide gel electrophoresis (PAGE) showed a single band of approximately 60 kd.

FITC labeling was carried out by adding 88 μg FITC in 1.7 μl dimethylformamide to a mixture of 1 ml fiber protein solution and 1 ml 0.2 M carbonate-bicarbonate buffer, pH 9.5. The mixture was kept at room temperature for 5 hr in the dark. Subsequently, free FITC was removed by gel filtration on sephadex G25.

Preparation of Human Peripheral Blood Mononuclear Cells

Human peripheral blood mononuclear cells (PBMC) were isolated from healthy blood donors by centrifugation on Ficoll density gradient (Sigma, St. Louis, MO) at 700 *g* for 30 min. The cell fraction was washed by low-speed centrifugation (400 *g*) in Hanks' balanced salt solution to remove platelets. Mononuclear cells were resuspended in Dulbecco's MEM (DMEM) supplemented with 10% FCS, stimulated with 1 μg/ml of phythemagglutinin (PHA) and 25 IU/ml interleukin-2 (IL-2), and incubated at 37°C in a 5% CO_2-humidified incubator in 24 well tissue culture plates. In preliminary studies, we had analyzed the proliferation kinetics in the presence of several media (RPMI, MEM, DMEM) in order to determine optimal conditions for lymphocyte cultivation. The highest rate of cell proliferation was observed when cells were grown in DMEM supplemented with both PHA and IL-2.

Flow Cytometry

Flow cytometry was carried out using a Becton-Dickinson (Mountain View, CA) fluorescence-activated cell sorter. Estimation of binding of Ad fiber was analyzed using FITC-labeled fiber protein. The cell subset analysis of PBMC preparations was undertaken with monoclonal anti-human CD3-, CD4-, and CD8 R-phycoerythrin (PE) conjugate purified mouse immunoglobulin (Sigma).

Fluorescence signals from 10^6 PBMC were obtained, and the percentage of positive cells labeled with PE and/or FITC was recorded. Briefly, cells were washed with PBS. The pellet was incubated on ice for 10 min, and 10 μl of FITC-labeled fiber protein was added. The suspension was gently mixed and incubated on ice for a further 30 min. Cells were washed with cold PBS and resuspended in PBS containing 0.5% paraformaldehyde. Storage of samples for 14 days was possible. For blocking experiments, cells were preincubated with self-prepared polyclonal antibodies against Ad 2 for 30 min on ice. For double labeling, cells were incubated with PE-conjugated monoclonal antibodies against lymphocyte surface protein marker for 30 min, followed by incubation with FITC-labeled Ad fiber protein and subsequently by the same procedure as described above. The data were analyzed by the program Lysis II.

TABLE I. Binding Activity of the FITC-Labeled Ad 2 Fiber Protein to FL Cells and Inhibition by Antibodies Against Ad Type 2 Using Flow Cytometric Analysis

Ad fiber protein (μl)	Treatment[a]	% of Membrane fluorescence (positive cells)
20	None	56.78 ± 2.53
20	+ Serum (1:100)	8.24 ± 1.83
	– Serum (1:100)	44.8 ± 4.37
40	None	90.77 ± 2.53
40	+ Serum (1:100)	14.31 ± 2.71
	– Serum (1:100)	82.73 ± 3.16

[a]Treatment of FL cells was with an equal volume of PBS (none), anti-Ad antibody-negative serum (– serum), or anti-Ad antibody-positive serum (+ serum) for 30 min at 4°C. Incubation of cells with FITC-Ad fiber protein was also for 30 min at 4°C.

PCR Assay

For PCR amplification, hexon-oligonucleotide primers of the regions 18858–18883 and 19136–19158 were used [Allard et al., 1990]. Nucleotide sequences were 5′-GCCGCAGTGGTCTTACATGCACATC-3′ and 5′-CAGCACGCCGCGGATGTCAAAGT-3′. The final amplification products were run on a 1% agarose gel containing ethidium bromide and visualized with UV light.

Stimulated PBMC were infected with two multiplicities of infection (moi) of Ad type 2, 8.9×10^3 and 8.9×10^2 particles per cell [Liebermann and Mentel, 1994]. After adsorption for 1 hr at 37°C, unbound virus was removed by washing with PBS three times. Cells were incubated in DMEM supplemented with 2% FCS at 37°C in a 5% CO_2-humidified atmosphere. DNA was extracted from cultured, infected PBMC using phenol-chloroform extraction. Samples were mixed with PCR reagents, including two amplification primers and Taq polymerase, and incubated in a thermocycler. Thirty amplification cycles were carried out in GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT). The three step profile consisted of 94°C for 10 sec, 56°C for 90 sec, and 72°C for 90 sec. Control samples for the detection of accidental contamination contained all reagents except for DNA; further DNA samples from uninfected cells were included.

RESULTS

A direct virus receptor assay was established. Ad fiber preparation was covalently labeled with FITC. The usefulness of this labeled preparation for binding studies was characterized on a permissive cell system, and samples of 10^6 FL cells were incubated for 30 min with this preparation. The results indicate that approximately 90% of cells showed membrane fluorescence (Table I). Treatment of cells with antibodies against Ad 2 influenced binding. Adsorption was significantly decreased, whereas preincubation with anti-Ad-negative

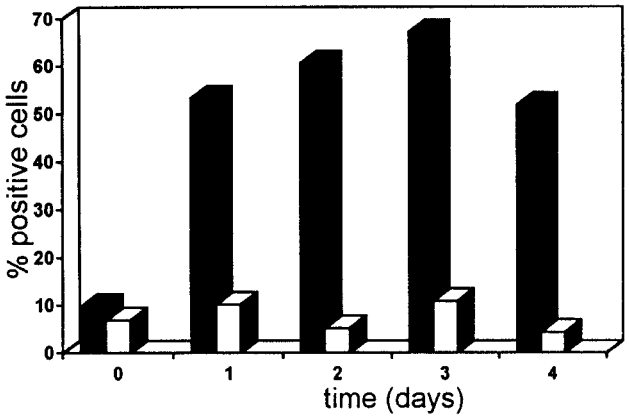

Fig. 1. Kinetics of adsorption of FITC-labeled Ad fiber to stimulated PBMC. Binding ability was detected after 1–4 days. Cells were incubated with FITC-labeled fiber protein in the presence □ or in the absence ■ of antibodies against Ad type 2.

serum was without effect. Inhibition of the anti-Ad antibody-positive serum by 85% is evidence of specific binding of fiber protein. After establishing the direct virus receptor assay, the interaction between Ad and PBMC was investigated using this fiber preparation. The recognition of receptor binding sites of the virus fiber on the surface of cells was explored on freshly isolated native PBMC and in cultures of these cells with and without stimulation. Cells in the native stage showed only few fluorescence signals (Fig. 1). After stimulation for 24 hr, a positive fiber attachment was observed in 53% of the population. To substantiate this finding, competition experiments were carried out using antibodies against Ad fiber protein and unlabeled fiber protein. Incubation of lymphocytes with antibodies blocked significantly the adsorption of fiber, the inhibition ranging 80–90%. The same effect was obtained using unlabeled fiber protein (Table II).

To examine which subsets of human peripheral blood cells are involved in these Ad fiber cell interactions, binding experiments were continued using double labeling of PBMC. The results of incubation with PE-labeled antibodies against CD3 surface molecules and FITC-labeled Ad fiber are shown in Figure 2. Of the cell population, 80% was identified as CD3-positive. After stimulation for 48 hr, nearly all T cells showed adsorption of FITC-labeled fiber protein.

Differentiation of the T lymphocyte subsets revealed that the freshly isolated cells consisted of approximately 50% CD4-positive cells and 35% CD8-positive cells (Figs. 3, 4). Incubation of PBMC with mitogen stimulation led to Ad fiber binding in nearly all cells defined as CD4 in the samples after 48 hr (Fig. 3).

In parallel studies, the role of CD8 cells was analyzed. Treatment of cells with mitogen resulted in receptor expression for Ad and approximately 70% of the CD8 subset showed fiber protein binding after 48 hr (Fig. 4).

To determine whether attachment of the Ad to the cell receptor of PBMC is followed by the subsequent

TABLE II. Effect of Preincubation With Unlabeled Fiber Protein, Anti-Ad Antibody-Negative Serum, and Anti-Ad Antibody-Positive Serum on the Attachment of FITC-Labeled Fiber Protein to PBMC After Cultivation for 24 hr

Inhibitor	Percentage inhibition
PBS (sham)	0
Unlabeled fiber protein	78.86
Anti-Ad antibody-positive serum	80.96
Anti-Ad antibody-negative serum	16.66

The inhibitor was added to the cell pellet and incubated for 30 min at 4°C. After washing three times with PBS, FITC-labeled Ad fiber protein was added. The binding assay was performed as described in text. Percentage of inhibition was calculated as follows: % positive cells with FITC-Ad – % positive cells with FITC-Ad and inhibitor/% positive cells with FITC-Ad × 100.

step of internalization, cells were infected with Ad. After incubation for 24, 48, and 72 hr, DNA was estimated using PCR. It was clearly demonstrated by PCR that the cells which are positive in binding studies also show positive signals for Ad DNA depending on time of cultivation and moi (Fig. 5), and the first signal was obtained after incubation for 48 hr.

DISCUSSION

Ad groups and serotypes differ considerably in their tissue specificity and virulence. Pathogenesis and the molecular basis is not fully understood, with AD causing self-limited and persistent infections [Evans, 1958; Routes et al. 1993]. The identity of cells serving as reservoirs for Ad is unknown, though tonsils and adenoids harbor AD [Horvath et al., 1986; Neumann et al., 1987]. Cells of the hematopoietic system play an important role in defense against infection.

The present studies were undertaken to elucidate the attachment of Ad to PBMC and to identify the subtypes which are involved. In regard to the importance of the medium for the membrane constitution and,

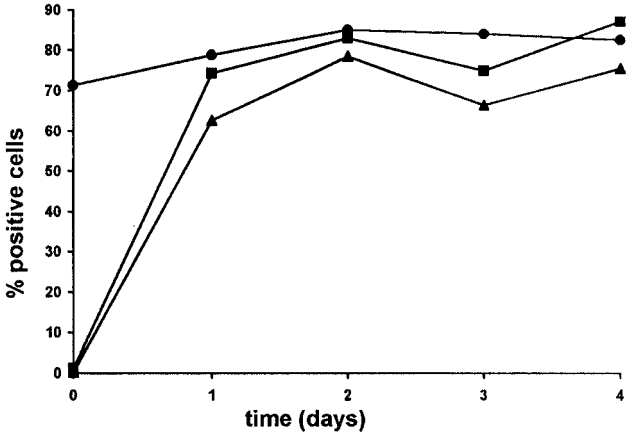

Fig. 2. Binding profile of Ad fiber protein to PBMC using flow cytometric analysis of in vitro cultivated cells. The whole fraction of PBMC was infected with Ad in vitro. Double staining was performed with FITC-labeled Ad fiber protein and PE-labeled anti-CD3 antibodies. Populations of PBMC were identified as $CD3^+$ cells (●), Ad fiber binding cells (■), and the part of CD3 cells with Ad fiber binding (▲).

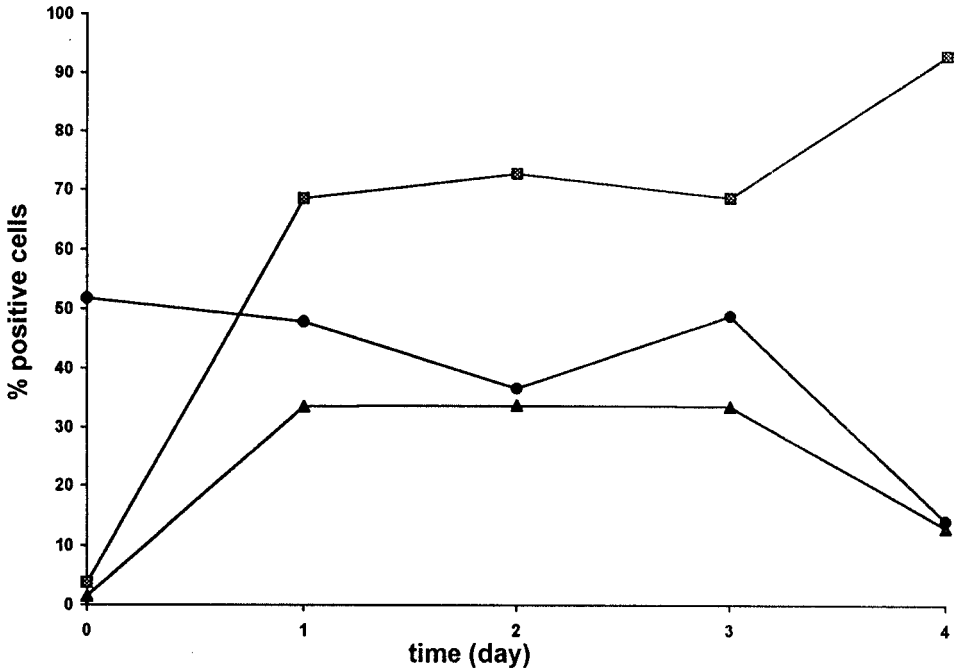

Fig. 3. Binding profile of Ad fiber protein to PBMC using flow cytometric analysis of in vitro cultivated cells. The whole fraction of PBMC was infected with Ad in vitro. Double staining was performed with FITC-labeled Ad fiber protein and PE-labeled anti-CD4 antibodies. Populations of PBMC were identified as $CD4^+$ cells (●), Ad fiber binding cells (■), and the part of CD4 cells with Ad fiber binding (▲).

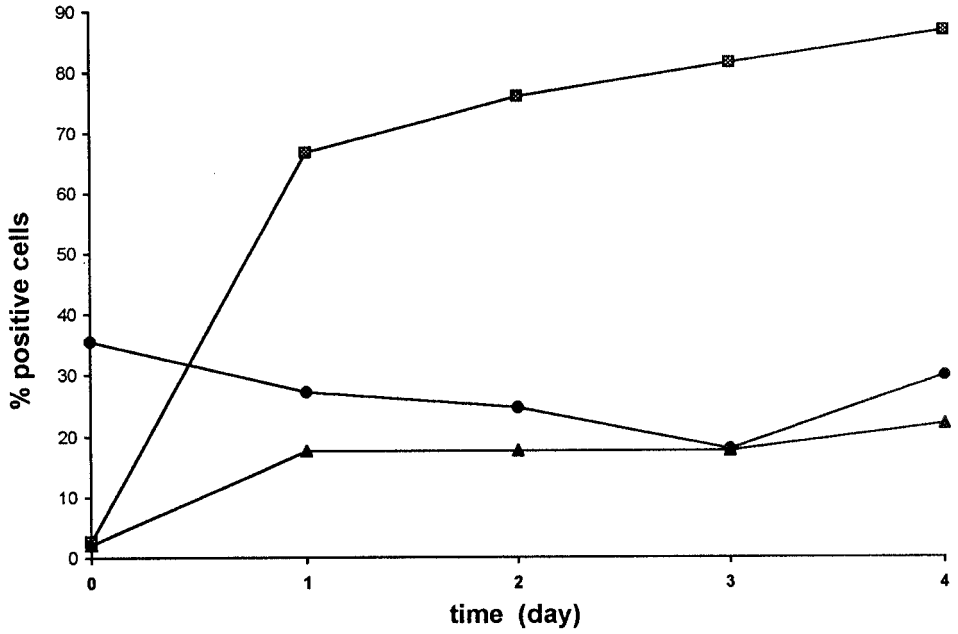

Fig. 4. Binding profile of Ad fiber protein to PBMC using flow cytometric analysis of in vitro cultivated cells. The whole fraction of PBMC was infected with Ad in vitro. Double staining was performed with FITC-labeled Ad fiber protein and PE-labeled anti-CD8 antibodies. Populations of PBMC were identified as $CD8^+$ cells (●), Ad fiber binding cells (■), and the part of CD8 cells with Ad fiber binding (▲).

therefore, for the process of attachment [Blixt, 1993], we analyzed different media to find optimal conditions. The highest cell proliferation rate in the experiments was estimated using DMEM.

Comprehensive experiments on the interactions of Ad and permissive permanent cells had been carried out [Defer et al., 1990; Belin and Boulanger, 1993]. There is evidence that the entry of Ad into permissive cells is connected with two separate cell receptors. Wickham et al. [1993] have shown that Ad type 2 binds to an unknown receptor by the fiber protein and to integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ through the penton base. The

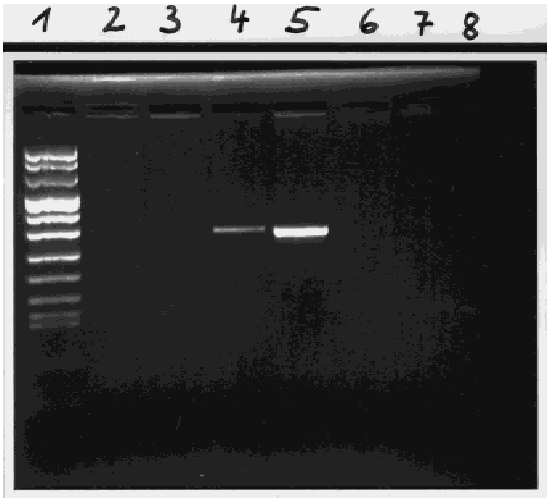

Fig. 5. Ad DNA detection in in vitro infected PBMC. PCR was performed with a specific primer pair amplifying a 300 bp segment from the hexon region. Shown are results of DNA samples derived from cells several days following in vitro infection. The yielded amplification products are identified on agarose gel. Cells were infected with two multiplicities of Ad (A = moi 8.9×10^3 and B = moi 8.9×10^2 virus particles per cell). Cultures were harvested 24 hr (lane 3, A; lane 6, B), 48 hr (lane 4, A; lane 7, B), and 72 hr (lane 5, A; lane 8, B) after infection. Cells were also studied immediately after infection (lane 2). Lane 1: DNA molecular weight marker VIII, 19–1114 bp (Boehringer, Mannheim, Indianapolis, IN).

binding of the penton base to the integrins seems to be necessary for the penetration step. The binding element of Ad in the attachment is the fiber structure. The head domain of the fiber participates in this process with primary plasma cell membrane receptors [Stevenson et al., 1995]. Different receptors are also described for other viruses. For example, herpes simplex virus glycoprotein C binds to heparan sulfate, while glycoprotein D binds to a protein molecule on the cells. Multiple receptors could be coreceptors and act together to modulate each other or to contribute complementary functions. Binding of the virus on the first receptor causes changes in the virus or host which enable the second receptor to bind [Haywood, 1994].

In contrast to permissive systems, there is little information about the interactions of Ad with cells of the hematopoietic system. Our binding studies were carried out using isolated fiber protein. The results clearly demonstrated that PBMC after mitogenic stimulation are able to upregulate significantly receptors for Ad binding. The specificity of this interaction was shown by inhibition experiments with antibodies against Ad and unlabeled fiber protein. These findings are interesting in relation to the establishment of viral persistence. The entry of Ad via interaction with cells of the immune system could be involved in this process. A reasonable scenario for persistent infections could be that lymphocytes and perhaps other cell types are a reservoir for Ad and the viral immune-modulating functions allow for ongoing low-level infections. There is evidence that Ad E3 proteins prevent cytolysis by cytotoxic T cells (CTL) and tumor necrosis factor (TNF). Two proteins of this region counteract with the host's antiviral defense, gp 19 K against CTL and 14.7 K against TNF [Körner et al., 1992; Stewart et al., 1995].

In the present study, kinetic experiments were undertaken to examine the internalization of Ad into PBMC. Using PCR amplification, we demonstrated Ad DNA in stimulated cells 48 hr after infection. Attachment of virus correlated to entry of virus into the cells depending on the infectious dose.

The findings suggest that resting cells are not permissive as demonstrated by Silver and Anderson [1988] who were unable to detect AD 2 receptors on human primary blood lymphocytes. Our results under the conditions of mitotic division suggest that lymphocytes belong to the host cells of Ad. The studies of Horvath and Weber [1988] showed that stimulation can only marginally lift the nonpermissiveness of PBMC.

We conclude that PBMC could play a role in infections by Ad, and on the basis of our results, further investigation on the identification of cell receptors for Ad on lymphocytes are indicated. More information about the interaction of Ad and lymphocytes, including complete Ad, substructures, and isolated membrane proteins of lymphocytes, are of importance in persistence and for the use of Ad as a vector for gene delivery.

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